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ANA PAULA ARSSUFI AMES

EFEITOS DO β-CARIOFILENO, O PRINCIPAL COMPONENTE DO ÓLEO DE COPAÍBA, SOBRE A INFLAMAÇÃO E O ESTRESSE OXIDATIVO EM RATOS ARTRÍTICOS

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Dissertação apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Mestre em Ciências Biológicas.

Orientador: Dr. Jurandir Fernando Comar.

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BIOGRAFIA

Ana Paula Arssufi Ames, filha de Leomar Antônio Ames e Edna Aparecida Arssufi Ames nasceu em Maringá/PR no dia 7 de abril de 1993. Possui graduação em Bioquímica pela Universidade Estadual de Maringá (2015). Durante a graduação foi contemplada com três bolsas de Iniciação Científica (CNPq), e neste período participou do trabalho intitulado como: *Anti-Inflammatory and antioxidant actions of copaiba oil are related to liver cell modifications in arthritic rats*, na área de metabolismo hepático e bioenergética, que lhe rendeu o primeiro artigo publicado. No mestrado desenvolveu a pesquisa com o composto isolado do óleo de copaíba, em consonância com o primeiro artigo.

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Todos os dias sou grata a Deus por tudo, e em relação a este trabalho não seria diferente. Não teria conseguido alcançar meus objetivos sem a benção divina para me dar forças e acalmar-me nos dias mais turbulentos.

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Aos meus pais eu devo a vida, o legado e a garra que me ensinaram a ter, não tenho como expressar a quão grata sou, sem os seus ensinamentos e exemplo de vida eu não estaria onde estou hoje.

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“O período de maior ganho em conhecimento e experiência é o período mais difícil da vida de alguém. ”

(Dalai Lama)

APRESENTAÇÃO

Este é um trabalho realizado no Laboratório de Metabolismo Hepático e Radioisótopos do Departamento de Bioquímica, no Laboratório de Inflamação do Departamento de Farmacologia e Terapêutica e no Laboratório de Histologia Animal do Departamento de Ciências Morfológicas da Universidade Estadual de Maringá, apresentado na forma de artigo científico original, em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas.

Artigo:

Ana Paula Ames Sibin, Camila Lima Barizão, Cristiane Vizioli Castro Ghizoni, Francielli Maria Souza Silva, Anacharis Babeto Sá Nakanishi, Lívia Bracht, Ciomar Aparecida Bersani Amado, Maria Raquel Marçal Natali, Adelar Bracht, Jurandir Fernando Comar. **Systemic inflammation and oxidative stress in arthritic rats are reduced by β-caryophyllene, the major component of copaiba oil.** Journal of Cellular Biochemistry, a ser submetido.

RESUMO GERAL

INTRODUÇÃO: A artrite reumatoide é uma doença inflamatória crônica de natureza autoimune que afeta as articulações e atinge 0,5-1,0% da população adulta mundial. A fisiopatologia da doença envolve uma intensa hiperplasia da cartilagem e da membrana sinovial articular com acentuada produção de espécies reativas de oxigênio (ROS), as quais atuam como mediadores dos danos teciduais. A artrite reumatoide é sistêmica e afeta diversos órgãos, como cérebro, pulmão e coração. A artrite induzida por adjuvante é uma imunopatologia experimental em ratos que compartilha muitas características com a artrite reumatoide. Além das articulações, outros órgãos dos animais são afetados, como o fígado, que apresenta alterações metabólicas e morfológicas associadas com um acentuado estresse oxidativo. O β-cariofileno é um sesquiterpeno natural encontrado em vários óleos essenciais ao qual tem sido atribuído atividade anti-inflamatória. O mesmo é o principal constituinte do óleo de copaíba, um fitoterápico da Amazônia brasileira, que diminuiu a inflamação e o estresse oxidativo sistêmicos de ratos artríticos, mas que causou modificações no metabolismo e morfologia hepática em ratos saudáveis (trabalho publicado previamente em nosso laboratório). O β-cariofileno parece ser o responsável pelos efeitos anti-inflamatórios do óleo de copaíba em ratos artríticos, mas sua ação sobre o fígado ainda é incerta. Desta forma, o presente estudo investigou a ação do β-cariofileno administrado oralmente sobre a inflamação sistêmica, o estresse oxidativo e o metabolismo hepático em ratos com artrite induzida por adjuvante. Em adição, este estudo fez uma comparação entre os efeitos do β-cariofileno com os efeitos já conhecidos do óleo de copaíba em ratos artríticos. **MÉTODOS:** A indução da artrite foi feita em ratos *Holtzman* (180-200g) por meio da administração do adjuvante completo de Freund. Os ratos foram distribuídos em nove grupos: controle, os quais receberam salina; controle negativo, os quais receberam óleo de milho; controle tratado com β-cariofileno nas doses de 215 e 430 $mg \cdot Kg^{-1}$; artrítico (salina); artrítico (óleo de milho); artrítico tratados com β-cariofileno nas doses 215 e 430 $mg \cdot Kg^{-1}$; e artrítico (controle positivo) tratados com ibuprofeno (30 $mg \cdot Kg^{-1}$). O tratamento foi feito diariamente por gavagem, o qual foi iniciado 5 dias antes da indução da artrite e perdurou por adicionais 18 dias após a indução. A dose de β-cariofileno corresponde a mesma quantidade presente nas doses de óleo de copaíba empregada para tratar os ratos

artríticos em um estudo prévio. O volume das patas e as lesões secundárias foram monitorados. O peso do fígado e linfonodos foram avaliados. E ainda, o número de leucócitos no sangue e recrutados para cavidade articular femorotibial de ratos artríticos foram determinados. No 19° dia, a cavidade peritoneal dos ratos previamente anestesiados foi exposta, o sangue colhido da veia cava e o fígado removido e dividido em duas partes: uma usada para o processamento histológico e outra para a preparação do homogenato. Proteínas carboniladas e ROS foram quantificados no homogenato para avaliar o estresse oxidativo hepático. O conteúdo de glutationa oxidada (GSSG) e reduzida (GSH), assim como a atividade da catalase, mieloperoxidase (MPO) e superóxido dismutase (SOD) foram determinados no homogenato. Também foram determinados a capacidade antioxidante total (TAC), tióis, proteínas carboniladas e atividade da MPO no plasma. A atividade das enzimas AST, ALT e fosfatase alcalina (ALP) foram determinadas no plasma para avaliar dano hepático. A análise morfológica e morfométrica do fígado foi feita através de imagens da região próxima à veia central do parênquima hepático. A gliconeogênese foi avaliada utilizando os fígados em perfusão isolada. A respiração e a geração de ROS foram adicionalmente determinadas em mitocôndrias isoladas do fígado. **RESULTADOS:** Os ratos artríticos apresentaram uma resposta inflamatória intensa ao adjuvante tanto na pata injetada quanto na pata contralateral. Estes animais também não ganharam peso corporal e apresentaram manifestações inflamatórias sistêmicas, como evidenciado pela aumentada atividade da MPO plasmática e hepática, lesões severas secundárias à artrite, peso elevado dos linfonodos e maior número de leucócitos articulares e circulantes. Nos ratos artríticos, as duas doses do β-cariofileno diminuíram o edema das patas injetadas, o peso dos linfonodos poplíteo e o número de leucócitos articulares e circulantes. Ratos artríticos não tratados apresentaram elevados níveis de ROS no fígado e de proteínas carboniladas no plasma e fígado, diminuição na atividade da catalase e no conteúdo de GSH no fígado, e redução de TAC e tióis no plasma. O β-cariofileno (430 $mg \cdot Kg^{-1}$) foi capaz de diminuir os níveis de proteínas carboniladas e a atividade da MPO no fígado e no plasma de animais artríticos. As duas doses do β-cariofileno diminuíram em 30% os níveis de ROS e reestabeleceram os níveis de GSH no fígado de ratos artríticos. A gliconeogênese hepática foi diminuída em 40% pela artrite e o tratamento não modificou este parâmetro. As atividades de AST e ALP foram maiores em ratos

artríticos e o tratamento com β-cariofileno diminuiu e normalizou estes parâmetros. A gliconeogênese e o peso corporal não foram modificados pelo tratamento dos animais controles. Animais artríticos também apresentaram menor número de hepatócitos por área de fígado (-23%) associado a um maior peso de fígado (+50%) e área dos hepatócitos (+18%), os quais não foram modificados pelo tratamento dos animais controles e artríticos. A produção de ROS e a respiração de mitocôndrias isoladas não foram modificadas pela patologia nem pelos tratamentos. **DISCUSSÃO E CONCLUSÃO:** O β-cariofileno e o óleo de copaíba (resultados já publicados) reduziram na mesma proporção o edema nas patas injetadas (30%) e contralateral (50%) (maiores doses) e, na dose menor, a atividade da MPO plasmática (30%). Essas ações semelhantes mostram que o β-cariofileno parece ser responsável por ações benéficas do óleo de copaíba (*C. reticulata*) em ratos artríticos. O β-cariofileno, além disso, reduziu a atividade da MPO do fígado, o número de leucócitos no sangue periférico e nas articulações. A atividade da MPO hepática não foi modificada e os leucócitos circulantes e articulares não foram quantificados para o óleo de copaíba. O β-cariofileno e o óleo de copaíba, ambos na maior dose, reduziram os níveis de proteínas carboniladas e ROS no fígado artrítico para valores próximos aos dos controles. Além disso, em ambas as doses, eles aumentaram a GSH no fígado artrítico para níveis próximos dos controles. Estes resultados sugerem que o β-cariofileno é responsável pela ação antioxidante do óleo de copaíba. Em adição, os resultados indicam que o β-cariofileno melhorou o estado oxidativo de ratos artríticos por meio dos seguintes mecanismos: diminuindo a inflamação, estimulando o sistema antioxidante endógeno e agindo diretamente como “varredor” radicais livres. O β-cariofileno mostra, além disso, atividade hepatoprotetora. No entanto, o sesquiterpeno não melhorou a hipertrofia dos hepatócitos, gliconeogênese, hepatomegalia e peso corporal, todavia, nem mesmo o ibuprofeno, a indometacina e a dexametasona melhoraram esses parâmetros (dados da literatura). Por outro lado, ao contrário do óleo de copaíba (dados publicados pelo nosso grupo de pesquisa), esses parâmetros não foram modificados em ratos saudáveis tratados e, portanto, mostram que o β-cariofileno não foi hepatotóxico.

Palavras-chave: artrite induzida por adjuvante, β-cariofileno, óleo de copaíba, estresse oxidativo, gliconeogênese, metabolismo hepático.

GENERAL ABSTRACT

BACKGROUND: Rheumatoid arthritis is an autoimmune and chronic inflammatory disease that affects primarily the joints and occurs in 0.5-1.0% of the adult population worldwide. The pathophysiology of arthritis involves an intense cytokine-mediated hyperplasia of the articular synovial membrane and cartilage and overproduction of reactive oxygen species (ROS), which act as mediators of tissue injury. Rheumatoid arthritis is a systemic disease and many organs are affected, such as brain, lungs and heart. Adjuvant-induced arthritis is an experimental immunopathology in rats that shares many features with rheumatoid arthritis. These animals present systemic inflammation and in addition to articular sites other organs are affected, as the liver, which presents morphological and metabolic alterations associated with a pronounced oxidative stress. β-Caryophyllene is a natural sesquiterpene found in various essential oils and it has been reported to present anti-inflammatory activity. β-Caryophyllene is the major component of copaiba oil, a Brazilian Amazon herbal medicine, which improved the systemic inflammation and oxidative status in arthritic rats, but caused harmful modifications in the liver cells metabolism and morphology of healthy rats (data previously published by our researchers). β-Caryophyllene seems to be responsible for the anti-inflammatory action of copaiba oil on arthritic rats, but its action is still uncertain, and it might even be one of the responsible for the harmful actions of the oil, although hepatoprotective action has been attributed to it. Therefore, the present study investigated the action of orally administrated β-caryophyllene on the systemic inflammation, oxidative status and liver cell metabolism in rats with adjuvant-induced arthritis. In addition, this study aimed to compare the effects of β-caryophyllene with those previously verified for copaiba oil. **METHODS**: The induction of arthritis was performed in *Holtzman* rats (180-200 g) with Freund's adjuvant. The rats were distributed into nine groups: controls, to which saline solution was administered; negative controls, to which corn oil was administrated; treated controls (Cβ 215 and 430), which were treated with β-caryophyllene at the doses of 215 and 430 $mg \cdot Kg^{-1}$; arthritic rats (saline); arthritic rats (corn oil); treated arthritic rats (Aβ 215 and 430), which received 215 and 430 $mg \cdot Kg^{-1}$ β-caryophyllene; and positive controls (AIBU), arthritic rats treated with ibuprofen (30 $mg \cdot Kg^{-1}$). The rats were daily treated by oral administration for 5 days prior and 18 days after of the arthritis induction. The doses

of β-caryophyllene correspond to the same amount present in the doses of copaiba oil used in the treatment of the arthritic rats in previous study. Paw volume and secondary lesions were measured. The weight of liver and lymph nodes were evaluated. Circulating leukocytes and those recruited into the femorotibial joint cavities were further quantified in arthritic rats. At day 19, peritoneal cavity of anesthetized rats was exposed, blood collected from the cava vein and the liver was removed and divided into two parts: one was used for histological processing and the other used for liver homogenate preparation. Protein carbonyl groups and ROS content were measured in the homogenate to evaluate liver oxidative stress. Oxidized (GSSG) and reduced (GSH) glutathione content and catalase, myeloperoxidase (MPO) and SOD activity were performed in the homogenate. Total antioxidant capacity (TAC), thiol, protein carbonyl groups and MPO activity were also measured in the plasma. AST, ALT and alkaline phosphatase (ALP) activities were measured in the plasma to evaluate liver damage**.** Morphologic and morphometric analysis was done with images from the region near the central vein of liver parenchyma. Gluconeogenesis was measured in isolated perfused livers. It was additionally investigated the respiration and ROS production in isolated hepatic mitochondria. **RESULTS**: Arthritic rats developed an intense inflammatory response to adjuvant in the injected and contralateral paw. They also presented no body weight gain and systemic manifestations, as evidenced by the higher plasma and liver MPO activity, severe secondary lesions, increased weight of lymph nodes and a higher number of leukocytes in the blood and joints. In arthritic rats, both doses of β-caryophyllene decreased the injected paw edema, the swollen of popliteus lymph nodes and the number of circulating and articular leukocytes. Non-treated arthritic rats presented higher levels of ROS in the liver and protein carbonyl groups in the plasma and liver, reduced activity of catalase and GSH content in the liver, and decreased TAC and thiols in the plasma. β-Caryophyllene (430 $mg \cdot Kg^{-1}$) abolished the increases of protein carbonyl groups and MPO activity in the liver and plasma of arthritic rats. Both doses of β-caryophyllene decreased the levels of ROS and restored the GSH content in the arthritic liver. Hepatic gluconeogenesis was 40% lower in the arthritic liver and β-caryophyllene did not improve it. AST and ALP were increased by 100 and 150%, respectively, by arthritis and treatment with β-caryophyllene reduced these parameters close to the normal ones. Gluconeogenesis and body weight was not modified in treated control rats. Arthritic

rats also presented reduced number of hepatocytes per liver area (-23%) associated to higher liver weight (+50%) and increased hepatocyte area (+18%), which were all not modified by β-caryophyllene in both control and arthritic rats. Neither ROS production nor respiration of isolated mitochondria was modified by the pathology or the treatments. **DISCUSSION AND CONCLUSION**: β-Caryophyllene and copaiba oil (published data) reduced in the same proportion the edema in the injected (30%) and contralateral (50%) paws (at the highest dose) and, in the lowest dose, plasmatic MPO activity (30%). These similar actions show that β-caryophyllene seems to be responsible for these beneficial actions of copaiba oil (*C. reticulata*) in arthritic rats. β-Caryophyllene in addition reduced the liver MPO activity, the number of leukocytes in the peripheral blood and those recruited into the joints. The liver MPO activity was not modified and circulating and articular leukocytes were not measured for copaiba oil. In relation to oxidative state, β-caryophyllene and copaiba oil at the highest dose reduced the protein carbonyl groups and ROS content in arthritic liver to levels close to the control values. In addition, at both doses, they increased GSH in the arthritic liver to levels close to the control values. These results strongly suggest that β-caryophyllene is responsible for the antioxidant action of copaiba oil in arthritic rats. Besides that, the results indicate that β-caryophyllene had improved the oxidative status of arthritic rats by means of the following mechanisms: by decreasing the inflammatory process, by slightly stimulate the endogenous antioxidant system, and by acting as direct free radicals scavenger. β-Caryophyllene shows in addition hepatoprotective activity. On the other hand, β-caryophyllene did not improve the adjuvant-induced swelling of hepatocytes, gluconeogenesis, hepatomegaly and low body weight, however, even ibuprofen, indomethacin and dexamethasone did not improve these parameters (literature data). However, unlike copaiba oil, these parameters were not modified in healthy rats and, therefore, β-caryophyllene was not associated to hepatotoxicity. With this in view, β-caryophyllene may be a good candidate as an adjuvant to potentiate the effects of conventional treatment of rheumatoid arthritis or even a starting point for anti-inflammatory drugs development.

Keywords*:* adjuvant-induced arthritis, β-caryophyllene, copaiba oil, oxidative status, gluconeogenesis, liver metabolism.

Systemic inflammation and oxidative stress in arthritic rats are reduced by β-caryophyllene, the major component of copaiba oil

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Running head: Anti-inflammatory and antioxidant activity of β-caryophyllene

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ABSTRACT

The present study investigated the action of β-caryophyllene, the major component of copaiba oil, on the systemic inflammation, oxidative status and liver cell metabolism of rats with adjuvant-induced arthritis, a model for rheumatoid arthritis. In addition, this study aimed to compare the effects of β-caryophyllene with those previously verified for copaiba oil, which improved the systemic inflammation in arthritic rats but caused harmful modifications in liver cells of healthy rats (published data). For this purpose, *Holtzman* healthy and arthritic rats were orally treated during 18 days with β-caryophyllene at the doses of 215 and 430 $mg \cdot Kg^{-1}$, which correspond to the same amount of β-caryophyllene in the doses of copaiba oil previously used in published study. Both doses of β-caryophyllene decreased the paw injected edema, the swollen of lymph nodes and the number of circulating and articular leukocytes in arthritic rats. β-Caryophyllene (430 $mg \cdot Kg^{-1}$) abolished the increases of protein carbonyl groups and myeloperoxidase (MPO) activity in the liver and plasma of arthritic rats. Similarly, β-caryophyllene decreased reactive oxygen species (ROS) by 30% and restored the GSH content in the arthritic liver. These beneficial actions of β-caryophyllene occurred in the same extension of those with copaiba oil in a previous study (*C. reticulata*). In addition, β-caryophyllene reduced the plasmatic markers of liver damage in arthritic rats. Hepatic gluconeogenesis was 40% lower in the arthritic liver and β-caryophyllene did not improve it. Arthritic rats also presented a reduced number of hepatocytes per liver area (-23%) associated to higher liver weight (+50%) and increased hepatocyte area (+18%), which were all not modified by β-caryophyllene. β-Caryophyllene did not improve the adjuvant-induced swelling of hepatocytes, gluconeogenesis, hepatomegaly and body weight. The results reveal that β-caryophyllene improves the articular and systemic inflammation of arthritic rats and that it reduces the oxidative stress in the liver and plasma. In addition, unlike copaiba oil (as data published by our researcher group), these parameters were not modified in healthy control rats and, therefore, β-caryophyllene was not associated to hepatotoxicity.

Keywords*: adjuvant-induced arthritis, β-caryophyllene, copaiba oil, oxidative status, gluconeogenesis, liver metabolism.*

INTRODUCTION

β-Caryophyllene, or (-)-*trans*-caryophyllene, is a natural sesquiterpene found in various essential oils and it has been reported to present many pharmacological properties, such as anti-inflammatory, neuroprotective, antidepressant, anti-allergic, antioxidant and antitumor [Liu et al., 2014; Chang et al., 2013; Choi et al., 2013; Calleja et al., 2013; Bento et al., 2011; Passos et al., 2007; Fernandes et al., 2007; Cho et al., 2007]. The anti-inflammatory activity, however, has been the most investigated property of β-caryophyllene, which inhibited the carrageenan-induced edema and proinflammatory cytokines production in rats and mice [Fernandes et al., 2007; Passos et al., 2007], inhibited the LPS-induced edema and NF-κB activation in the paw of rats [Medeiros et al., 2007], and attenuated the intestinal inflammation in mice with dextran sulfate-induced colitis [Bento et al., 2011; Cho et al., 2007].

β-Caryophyllene is the major constituent of copaiba oil, an oleoresin extracted from plants of the genus *Copaifera* which is an important herbal medicine of the Brazilian Amazon commercialized worldwide [Desmarchelier, 2010]. β-Caryophyllene accounts for approximately 40% of the oil extracted from *Copaifera reticulata* (*C. reticulata*), the most abundant species of the Brazilian Amazon [Veiga Jr et al., 2007]. Copaiba oil also presents anti-inflammatory activity and, for this reason, it has been popularly used to treat rheumatic diseases [Desmarchelier, 2010; Veiga Jr et al., 2007]. In this regard, copaiba oil (*C. reticulata*) attenuated the pronounced systemic inflammation and oxidative stress in rats with adjuvant-induced arthritis, a model for human rheumatoid arthritis [Castro-Ghizoni et al., 2017].

Rheumatoid arthritis is a systemic autoimmune and chronic inflammatory disease that affects primarily the joints and occurs in 0.5-1.0% of the adult population worldwide [Uhlig et al., 2014]. The pathophysiology of arthritis involves an intense cytokine-mediated hyperplasia of the synovial membrane and overproduction of reactive oxygen species (ROS), which act as mediators of tissue injury [Mcinnes & Schett, 2011]. In addition to inflammation, oxidative stress is increased in the joints and systemically [Lemarechal et al., 2006]. Metabolic alterations are also prominent in rheumatoid arthritis, as the muscle wasting condition known as rheumatoid cachexia [Roubenoff, 2009]. Metabolic modifications are equally significant in the liver of rats with adjuvant-induced

arthritis, such as increased fatty acid oxidation, increased glycolysis and reduced gluconeogenesis [Wendt et al., 2018; Sá-Nakanishi et al., 2018; Fedatto et al., 1999]. Oxidative stress is also altered in the plasma, liver, brain and heart of arthritic rats [Bracht et al., 2016; Schubert et al., 2016; Wendt et al., 2015; Comar et al., 2013]. Particularly in the liver, where inflammation and metabolic alterations are prominent, oxidative stress is quite pronounced when compared to other organs [Wendt et al., 2015; Comar et al., 2013].

Adjuvant-induced arthritis is a model of experimental immunopathology in rats which exhibits a strong and generalized inflammatory response and, therefore, allows to investigate the pathogenesis effects in the joints and systemically, as modifications in the liver metabolism [Bendele et al., 1999]. This model should also allow extrapolations to patients with rheumatoid arthritis.

As β-caryophyllene is by far the major constituent of copaiba oil (*C. reticulata*), the anti-inflammatory activity of the oil has been attributed to it, however, other minor constituents have been reported to present anti-inflammatory activity, particularly the α-humulene [Leandro et al., 2012; Fernandes et al., 2007]. On the other hand, copaiba oil orally administrated inhibited the hepatic gluconeogenesis and altered the hepatic cell morphometry in healthy rats, effects that were attributed to a possible hepatotoxicity of the oil [Castro-Ghizoni et al., 2017]. Healthy rats treated with copaiba oil do indeed present alterations in hepatic morphology that were related to liver cholestasis and vascular congestion [Botelho et al., 2010; Brito et al., 2000]. It is unknown if either β-caryophyllene or the others components of copaiba oil is responsible for the functional and morphological modifications that occurred in the liver of healthy rats. However, hepatoprotective action has been attributed to β-caryophyllene, as verified in rats with carbon tetrachloride-induced hepatic injury and in mice with alcoholic steatohepatitis [Varga et al., 2018; Calleja et al., 2013;]. Moreover, the sesquiterpene also attenuated the cisplatin-induced nephrotoxicity in mice [Horváth et al., 2012]. These protective actions of β-caryophyllene were all associated with a decrease in the tissue oxidative stress.

Considering that β-caryophyllene seems to be responsible for the anti-inflammatory action of copaiba oil, particularly that in arthritic rats, and its action on the liver is still uncertain, the present study aimed to evaluate the actions of orally administrated β-caryophyllene on the articular and systemic

inflammation, the liver function and oxidative status in rats with adjuvant-induced arthritis. Liver involvement was assessed by means of hepatic morphometry and plasmatic parameters of liver damage. Gluconeogenesis was measured in perfused livers to evaluate the action of β-caryophyllene on the hepatic function of arthritic and healthy rats. Considering that β-caryophyllene has been reported to present antioxidant activity [Varga et al., 2018; Castro-Ghizoni et al., 2017; Calleja et al., 2013], this study has also evaluated the oxidative stress in the arthritic liver and the production of ROS in isolated hepatic mitochondria. β-Caryophyllene presents in its structure a cyclobutane ring and a trans-double bond in a nine-carbon ring (Fig. 1A), both rare in nature, which makes it a compound of singular interest, particularly as a starting point for the development of anti-inflammatory and anti-rheumatic drugs.

MATERIAL AND METHODS

Chemicals

(-)-trans-Caryophyllene (β-caryophyllene), o-phthalaldehyde (OPT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 2,4-dinitrophenylhydrazine (DNPH), oxidized dichlorofluorescein (DCF), reduced glutathione (GSH), oxidized glutathione (GSSG) and were purchased from Sigma Chemical Co®. (St. Louis, MO, USA). Commercial kits for AST, ALT, alkaline phosphatase, albumin and total proteins were purchased from Gold Analisa Diagnóstica® Ltda (Belo Horizonte, MG, Brazil). All other chemicals were of analytical grade.

Animals and induction of arthritis

Male *Holtzman* rats weighing around 170-180g (50 days old) were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and maintained under standard laboratory conditions at a temperature of 24±3ºC under a regulated 12h light/dark cycle. The animals were housed in conventional steel cages (3 rats/cage) and were fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil). For the induction of arthritis, animals (180-200g) were injected subcutaneously in the left hind paw with 0.1 mL (500 µg) of Freund's adjuvant (heat-inactivated *Mycobacterium tuberculosis*, derived from the human strain H37Rv), suspended in mineral oil [Donaldson et al., 1993]. Rats of similar ages served as controls. All procedures followed the guidelines of the Brazilian Council for the Control of Animal Experimentation (CONCEA) and were previously approved by the Ethics Committee for Animal Experimentation (CEUA) of the State University of Maringá (Protocol number CEUA 4643280817).

Experimental design

Fifty-four rats were randomly distributed into nine groups (n=6 per group): controls (C), to which saline solution was administered; negative controls (Ccorn), to which corn oil was administrated; treated controls (Cβ 215 and Cβ 430), which were treated with β-caryophyllene at the doses of 215 and

430 mg·Kg^{-1}; arthritic rats (A), to which saline solution was administered; arthritic rats (Acorn), to which corn oil was administrated; treated arthritic rats (Aβ 215 and Aβ 430), which received β-caryophyllene at the doses of 215 and 430 mg·Kg^{-1}; positive controls (AIBU), arthritic rats treated with ibuprofen at the dose of 30 mg·Kg^{-1}. The whole procedures were repeated three times to evaluate all parameters of this study. Rats were treated once a day in the morning by oral administration (gavage) of β-caryophyllene, corn oil, saline or ibuprofen for 5 days prior to the induction of arthritis and by additional 18 days. Daily doses of β-caryophyllene corresponded to the same amount of β-caryophyllene present in the copaiba oil doses previously used to treat arthritic rats [Castro-Ghizoni et al., 2017; Ziech et al., 2013].

Evaluation of the inflammatory response

Animals were weighed and evaluated for their adjuvant-induced inflammatory response over 18-days period. Following adjuvant inoculation, the volume of both hind paws up to the tibiotarsal joint was measured by plethysmography. The results were expressed in terms of increased paw volume in relation to the initial volume (volume at day 0). The appearance and severity of secondary lesions were also assessed from the 10th to the 18th day according to the following score graded from 0 to 5: (+1) appearance of nodules in the tail; (+1 or +2) appearance of nodules in one or both ears; and (+1 or +2) appearance of swelling in one or both forelimbs [Bracht et al., 2012]. Blood was collected by means of the tail incision to obtain the total and differential count of circulating leukocytes. Total and differential counts of leukocytes recruited into the femorotibial joint cavity were additionally performed at the 19th day as previously described [Estevão-Silva et al., 2016].

Blood collection and tissue preparation

Rats fasted for 12 h were deeply anesthetized with sodium thiopental (100 mg·kg^{-1}) plus lidocaine (10 mg·Kg^{-1}) and the peritoneal cavity was surgically exposed. Blood was then collected from the cava vein and deposited into tubes with 100 IU mL^{-1} of sodium heparin. The liver was subsequently removed and divided into two parts: one was immediately freeze-clamped and stored in liquid nitrogen for oxidative status assessment and the other was used for histological processing. Thereafter, the hind femorotibial joints were

surgically exposed, the articular cavities were washed with 40 µL of phosphate-buffered saline (PBS) solution containing 1mM EDTA and the exudates used for leukocyte count.

The blood was centrifuged at 3,000*g* for 10 min and the supernatant was separated as the plasmatic fraction. For preparing the liver homogenate, the freeze-clamped portion of tissue was homogenized in a Van Potter-Elvehjem homogenizer with 10 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and an aliquot was separated for use as total homogenate. The remaining homogenate was centrifuged at 11,000*g* for 15 min and the supernatant separated as a soluble fraction of the homogenate.

For histological processing, the liver samples were fixed in 10% Bouin solution, dehydrated in graded ethanol, cleared in xylol and embedded in paraffin blocks. Semi-serial 6 µm thick cross-sections of liver were prepared with a rotary microtome (Leica RM2245), mounted on a slide and stained with hematoxylin-eosin to determine the morphology and morphometry.

Plasmatic analytical assays

The total antioxidant capacity (TAC), protein sulfhydryl groups (thiols), protein carbonyl groups and myeloperoxidase (MPO) activity were measured in the plasma to evaluate the oxidative and inflammatory state. Albumin content and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were measured in the plasma to evaluate liver damage using commercial kits (Gold Analisa®). The activity of MPO was measured as the increase in absorbance due to the oxidation of o-dianisidine at 460 nm and the activity calculated using the molar extinction coefficient (ε) of $11.3x10^{3}\cdot M^{-1}\cdot cm^{-1}$ [Bradley et al., 1982].

The total antioxidant capacity (TAC) of the plasma was measured by spectrophotometry using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) or ABTS [Bracht et al., 2016]. TAC was calculated from the standard curve prepared with Trolox, a water-soluble analog of vitamin E, and the results were expressed as nmol ·(mL plasma)$^{-1}$.

Plasmatic thiol contents were measured by spectrophotometry (412 nm) using DTNB (5,5'-dithiobis 2-nitrobenzoic acid) as previously described [Faure & Lafond, 1995]. Thiol contents were calculated using the molar extinction coefficient (ε) of $1.36 \times 10^{4}\cdot M^{-1}\cdot cm^{-1}$ and expressed as nmol·(mL plasma)$^{-1}$.

Protein carbonyl groups were measured by spectrophotometry using 2,4-dinitrophenylhydrazine [Levine et al., 1990]. The levels of protein carbonyl groups were calculated using the molar extinction coefficient (ε) of 2.20×10^4 $M^{-1} \cdot cm^{-1}$ and expressed as $nmol \cdot (mL\ plasma)^{-1}$.

Liver oxidative stress parameters

The levels of carbonylated proteins were measured in the supernatant of liver homogenate by spectrophotometry with DNPH using the same procedure already described for the plasma [Levine et al., 1990].

The levels of reactive oxygen species (ROS) were quantified in the supernatant of liver homogenate by spectrofluorimetry with 2',7'-dichloro-fluorescein diacetate (DCFH-DA) [Siqueira et al., 2005]. The assay quantifies the oxidation of DCFH-DA to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The formation of DCF was measured after stopping the reaction with ice using a spectrofluorimeter RF-5301 (Shimadzu) with excitation and emission wavelengths at 504 and 529 nm, respectively. A standard curve with oxidized dichlorofluorescein (DCF) was used to express the results as $nmol \cdot (mg\ protein)^{-1}$.

Reduced (GSH) and oxidized glutathione (GSSG) were measured spectrofluorimetrically (excitation at 350 nm and emission at 420 nm) by means of the *o*-phthalaldehyde (OPT) assay as previously described [Hissin & Hilf, 1976]. Fluorescence was estimated as GSH. For the GSSG assay, the sample was previously incubated with 10 mM N-ethylmaleimide and subsequently with a mixture containing 1 M NaOH and 0.4 µM OPT to detect fluorescence. Standard curves were prepared with GSH or GSSG.

The activities of catalase, superoxide dismutase (SOD) and MPO were assayed in the supernatant of liver homogenate. The catalase activity was estimated by measuring the change in absorbance at 240 nm using H_2O_2 as substrate and expressed as $mmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$ [Bergmeyer, 1974]. The results were calculated using the molar extinction coefficient (ε) of $9.6\ x\ 10^{-3}$ $M^{-1} \cdot cm^{-1}$. The activity of SOD was estimated by its capacity to inhibit pyrogallol autoxidation in alkaline medium at 420 nm [Marklund & Marklund, 1974]. One SOD unit was considered the quantity of enzyme that was able to promote 50% inhibition and results were expressed as $units \cdot (mg\ protein)^{-1}$. The activity of

MPO was measured using the same procedure already described above for the plasma [Bradley et al., 1982].

Histological analyses

The morphologic and morphometric analysis is carried out using images from the region near the central vein of the liver parenchyma. Images were captured from an optical microscope (Olympus BX41®, Japan) with a QColor3® camera (Olympus American INC, Canada), coupled to the software Q-Capture®. Hepatocytes number and area were evaluated using the program Image-Pro Plus® 4.5 (Media Cybernetics). For the number of hepatocytes, 50 images per animal were counted in an area of 329,972.45 μm^2 per image, totaling 250 images per group. For quantifying the hepatocytes areas, 200 hepatocytes per animal were examined, totaling 1000 hepatocytes per group (μm^2).

Mitochondrial ROS production and respiration

Hepatic mitochondria were isolated by differential centrifugation [Saling et al., 2011]. Mitochondrial oxygen consumption was measured polarographically using a Teflon-shielded platinum electrode [Saling et al., 2011]. Mitochondria were incubated in the closed oxygraph chamber in a medium (2.0 mL) containing 0.25 M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM EDTA and 10 mM Tris-HCl (pH 7.4). Succinate and α-ketoglutarate, both at a concentration of 10 mM, were used as electron donor substrates for complex I and II, respectively, of the mitochondrial electron transport chain. ADP, for a final concentration of 0.125 mM, was added at appropriate times. Rates of oxygen consumption were computed from the slopes of the recorder tracings and expressed as $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. The respiration rates were measured under two conditions: (a) before the addition of ADP (substrate respiration or basal) and (b) just after ADP addition (state III respiration). The ADP/O ratio was determined according to Chance & Williams [1955].

The rate of mitochondrial ROS production (real-time ROS production) was estimated by measuring the linear fluorescence increase due to DCF formation [Biazon et al., 2016]. Briefly, intact mitochondria were suspended in 2 mL of a mixture containing 250 mM mannitol, 1.36 μM DCFA-DA, 10 mM Hepes buffer (pH 7.2), and 10 mM succinate as respiratory substrate. The reaction was initiated by addition of 0.4 μM horseradish peroxidase and the fluorescence was

recorded during 10 min under agitation. The results were expressed as $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

Liver perfusion and gluconeogenesis

Gluconeogenesis was measured in the perfused livers of 12 h fasted rats. Hemoglobin-free, non-recirculating liver perfusion was performed as previously described [Comar et al., 2003]. After cannulation of the portal and cava veins, the liver was removed and positioned in a plexiglass chamber. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment to 37ºC. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and it was adjusted to between 30 and 33 $mL \cdot min^{-1}$. The oxygen concentration in the venous perfusate was monitored by a teflon-shielded platinum electrode. After stabilization of oxygen consumption, L-lactate (2 mM) was added to the perfusion fluid as a gluconeogenic substrate. Samples of the effluent perfusion fluid were collected at two minutes intervals and analyzed for their content of glucose and pyruvate [Bergmeyer, 1974]. At the end of perfusion, the liver was removed and weighed to allow precise metabolic calculations.

Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of the GraphPad Prism Software (version 5.0). The statistical significance of the data was analyzed by means of ANOVA ONE-WAY and the Newman-Keuls post-hoc test was applied with the 5% level of significance ($p < 0.05$).

RESULTS

Effects of β-caryophyllene on induction and development of adjuvant arthritis

Table I shows the liver, lymph nodes weight, body weight and inflammatory parameters due to arthritis development. The initial volume of the hind paws before adjuvant injection was 1.43±0.03 mL. Inflammatory reactions in the injected paw were observed on the first day and they were equal in all arthritic groups (not shown). At day 18 the volume of the injected paw of arthritic rats had increased by 307% relative to its initial volume. This increase was considerably less pronounced in arthritic rats treated with 215 and 430 $mg \cdot Kg^{-1}$ β-caryophyllene (210%), or ibuprofen (174%). The volume of the contralateral paw of arthritic rats had increased by 160%. Treatment of arthritic rats with 430 $mg \cdot Kg^{-1}$ β-caryophyllene or ibuprofen diminished these increases to 70 and 84%, respectively. The dose of 215 $mg \cdot Kg^{-1}$ β-caryophyllene was ineffective. Secondary lesions appeared at day 10 and reached the highest scores at day 18. Only ibuprofen (positive control) decreased the score of arthritis at day 18.

The body weight was monitored before adjuvant injection and at the day 18 after the arthritis induction. The mean initial weight (minutes before arthritis induction) of the animals was 202±4.0 g. The body weight of non-treated controls was 44% higher at day 18. The body weight gain was similar in all controls groups. In practical terms non-treated and treated arthritic rats did not gain body weight during this period. The liver weight was similar in the non-treated and treated controls. The liver weight of non-treated arthritic rats was 50% higher (compared to the controls) and treatments did not modify it. The weights of the popliteus and inguinal lymph nodes (right plus left) were greatly increased in arthritic rats when compared to the controls. The treatment of control rats did not modify the lymph nodes, but the treatment of arthritic rats with 430 $mg \cdot Kg^{-1}$ β-caryophyllene decreased the weight of popliteus and inguinal lymph nodes by 50 and 42%, respectively (compared to the group arthritis).

At day 18, the number of total blood leukocytes in non-treated arthritic rats was more than four times higher than initially (day 0) and, in addition, polymorphonuclear leukocytes were predominant. Treatment of animals with

215 and 430 mg·Kg^{-1} β-caryophyllene or ibuprofen caused a decrease of approximately 40% in the number of total leukocytes in the blood. The number of total leukocytes recruited into the articular cavity (joint of the injected paw) was approximately twice higher when compared to that in the contralateral joint. Treatment of animals with ibuprofen or 215 and 430 mg·Kg^{-1} β-caryophyllene caused a decrease of approximately 77% of this parameter. Only ibuprofen decreased the number of leukocytes recruited into the femorotibial right joint. No difference was found between saline and corn oil treatment and, therefore, the data obtained with corn oil were omitted in the next results.

Liver oxidative stress and inflammatory status

The activity of MPO was higher in the liver of arthritic rats (37%) and 430 mg·Kg^{-1} β-caryophyllene or ibuprofen (positive control) decreased MPO activity to levels close to the control values (Fig. 1B). The levels of protein carbonyl groups in the liver were 40% higher in arthritic rats (compared to the controls; Fig.1C). The treatment with 430 mg·Kg^{-1} β-caryophyllene or ibuprofen decreased the protein carbonyl groups in arthritic rats to levels close to the control values. Treatment of controls did not modify the levels of hepatic protein carbonyls. Tissue oxidative injury is normally caused by ROS, whose liver contents were 100%' higher in the arthritic rats (compared to the controls; Fig.1D). The treatment of arthritic animals with 215 and 430 mg·Kg^{-1} β-caryophyllene or ibuprofen decreased the liver ROS contents by approximately 30%.

Plasmatic oxidative and inflammatory status

The activity of MPO, total antioxidant capacity (TAC), the levels of protein carbonyl and thiol groups are shown in Fig. 2. The activity of MPO was five times higher in the plasma of arthritic rats (compared to the controls; Fig. 2A). The treatment of arthritic rats with 215 and 430 mg·Kg^{-1} β-caryophyllene or ibuprofen decreased the MPO activity by approximately 35% in the plasma of arthritic rats. The levels of protein carbonyl groups, a pro-oxidant parameter, were 50% higher in the plasma of non-treated arthritic rats (compared to the controls; Fig 2C). The treatment of arthritic rats with β-caryophyllene at the doses of 215 and 430 mg·Kg^{-1} decreased the levels of carbonyl protein groups (-20%). Plasmatic TAC and thiol groups, two antioxidant parameters, were 38

and 53% lower in arthritic animals. Only treatment with 430 mg·Kg^{-1} β-caryophyllene increased plasmatic TAC in arthritic rats. The treatment of arthritic rats did not modify the plasmatic thiol groups. Treatment of the controls animals with β-caryophyllene did not cause any changes in the plasma oxidative state.

Liver antioxidant status

The levels of glutathione and the activities of catalase and SOD are shown in the upper half of Table II. The levels of GSH in the liver of arthritic rats were 63% lower of those in the controls. The treatment of arthritic rats with 215 and 430 mg·Kg^{-1} β-caryophyllene or ibuprofen increased GSH to levels close to the control values. Treatment of controls did not modify the levels of hepatic GSH. The levels of GSSG in the liver of arthritic rats were 150% higher of those in the controls. The treatment of arthritic rats with β-caryophyllene or ibuprofen decreased GSSG to levels close to the control values. The GSH/GSSG ratio in the arthritic liver was only one-fifth of that in the controls and treatment with 430 mg·Kg^{-1} β-caryophyllene or ibuprofen increased this value by 280 and 210%, respectively. The catalase activity in the liver of arthritic rats was only 23% of that in the control rats. Treatment of control and arthritic rats did not modify the catalase activity. The SOD activity was reduced in 42% by the adjuvant and only ibuprofen reversed this.

Plasmatic biochemical parameters

The plasmatic AST, ALT and ALP activities were measured to evaluate if the treatment with β-caryophyllene causes liver damage. Plasmatic albumin levels were assessed as a parameter of systemic inflammation. The results are shown in the lower part of Table II. The arthritis induction increased the activity of plasmatic AST and ALP by 100 and 150%, respectively, and diminished the albumin levels (-30%). The treatment of arthritic rats with 215 and 430 mg·Kg^{-1} β-caryophyllene or ibuprofen decreased AST and ALP to levels close to the control values, but only ibuprofen (positive control) increased the levels of albumin in the plasma. Treatment of controls did not modify these parameters.

Respiration and ROS production in isolated hepatic mitochondria

Considering that the liver ROS content was modified by β-caryophyllene in

arthritic rats, the rate of ROS production (real-time ROS production) and the associated respiratory activity were measured in freshly isolated hepatic mitochondria. The results are shown in Fig. 3. Respiratory activity was measured in mitochondria of rats treated with β-caryophyllene only at the dose of 430 $mg \cdot Kg^{-1}$. No modification in the respiration and ROS production was verified in hepatic mitochondria of non-treated and treated control and arthritic rats.

Liver histology

Morphological and morphometric analyses were performed in the liver to evaluate if the increased liver weight in arthritic rats is associated with alterations in parenchyma structure. Only the dose of 430 $mg \cdot Kg^{-1}$ β-caryophyllene was used. Fig.4 depicts the morphology of liver sections stained with hematoxylin-eosin. Non-treated control rats showed a normal histological structure as noted by typical central vein and portal space, and hepatocytes arranged in cords with a central nucleus and preserved cytoplasm. Non-treated arthritic rats showed some alterations, as sinusoid dilatation, distortion of the normal architecture of hepatocytes and encapsulated inflammatory foci (yellow arrow in Fig.4). No modification in the hepatic morphology was verified in treated controls and only discreet modifications in treated arthritic rats, particularly lower encapsulated inflammatory foci when compared to non-treated arthritic rats. The results of the morphometric analysis are shown in the lower part of Fig.4. The number and area of hepatocytes were, respectively, 23% lower and 18% higher in non-treated arthritic rats (compared to the controls). Treatment with β-caryophyllene did not modify either the number or area of hepatocytes in both control and arthritic rats.

Gluconeogenesis in perfused livers

The gluconeogenic pathway is extremely sensitive to alterations in the cellular integrity and function [Soares et al., 2013]. Therefore, the effects of treatment with β-caryophyllene on gluconeogenesis were evaluated in perfused livers using as substrate L-lactate, which is one of the main gluconeogenic substrates in humans and rodents and in addition allows evaluating the complete gluconeogenic machinery from pyruvate up to glucose [Fedatto et al., 1999]. The upper part of Fig. 5 shows the time courses of glucose production, pyruvate production and oxygen consumption of non-treated and treated

control and arthritic rats, and illustrates the experimental protocol. The basal rates of glucose and pyruvate production were minimal and similar for all groups, however, the basal oxygen consumption was lower in treated and non-treated arthritic rats. After the introduction of L-lactate, all parameters were stimulated, but only glucose production was differently increased in control and arthritic rats. The lower part of Fig. 5 shows the increment in each parameter due to 2 mM L-lactate infusion. The values of increment were calculated as [final values at the end of the infusion period with L-lactate] - [basal rates before infusion of L-lactate]. The increment of glucose production was 41% lower in non-treated arthritic rats when compared to the controls. The treatment of arthritic rats did not improve the glucose production. The increment in glucose production was not different for treated and non-treated controls. The increment of oxygen consumption and pyruvate production was similar for all groups.

DISCUSSION

The experimental model of chronic inflammation used in the present study is considered a model of severe arthritis in rats and it shares features of severe rheumatoid arthritis [Stolina et al., 2009]. In this model, animals develop an intense inflammatory response to the adjuvant in the paws (polyarthritis) and present cachexia and generalized inflammatory manifestations [Stolina et al., 2009]. In this study, these manifestations were evidenced by the increased plasmatic and hepatic MPO activity, paw edema, severe secondary lesions to arthritis, increased weight of lymph nodes and increased number of leukocytes in the peripheral blood and into the joints. In addition, histological alterations, increased oxidative stress and reduced gluconeogenesis were verified in the liver of arthritic rats and corroborate previous findings [Sá-nakanishi et al., 2018; Castro-Ghizoni et al., 2017; Comar et al., 2013; Fedatto et al., 1999].

One purpose of this work was to evaluate the anti-inflammatory effect of β-caryophyllene on arthritis in rats and to compare it with the effect of copaiba oil (*C. reticulata*) previously verified for this same experimental model [Castro-Ghizoni et al., 2017]. Table III shows the values (%) of some parameters to compare the effects of β-caryophyllene and copaiba oil on arthritic rats. The sesquiterpene accounts for approximately 37% of the copaiba oil. The doses of β-caryophyllene in the present study (215 and 430 $mg \cdot Kg^{-1}$) were equivalent to the volume of β-caryophyllene present in the doses of the oil (0.58 and 1.15 $g \cdot Kg^{-1}$) used in the previous study drove by our researcher group [Castro-Ghizoni et al., 2017; Ziech et al., 2013].

β-Caryophyllene and copaiba oil reduced in the same proportion the edema in the injected and contralateral paws (at the highest dose) and, in the lowest dose, the weight of popliteus lymph nodes and plasmatic MPO activity. β-Caryophyllene, in addition, reduced the liver MPO activity (at the highest dose), the number of total leukocytes in the peripheral blood and those recruited into the left joints. The liver MPO activity was not modified and circulating and articular leukocytes were not measured for copaiba oil. Copaiba oil improved the edema in the contralateral paw at both dose, on the other hand, β-caryophyllene was effective only at the highest dose. These results show that the anti-inflammatory effects of copaiba oil were slightly higher of those of β-

caryophyllene, which can be attributed to some minor constituent of the oil acting synergistically. In this regard, α-humulene reduced the carrageenan-induced edema and the release of inflammatory mediators in the paw of rats [Fernandes et. al., 2007]. As these effects were equal to or less than those of β-caryophyllene and the α-humulene accounts for less than 5% of copaiba oil *(C. reticulata)*. β-Caryophyllene is not the only one but is the major anti-inflammatory constituent of this copaiba oil [Ziech et al., 2013; Fernandes et. al., 2007]. Some anti-inflammatory actions of copaiba oil were not verified with the highest dose and were previously attributed to the harmful action of the oil [Castro-Ghizoni et al., 2017]. On the other hand, both β-caryophyllene and copaiba oil did not improve either the secondary lesions (arthritic score) and body weight of arthritic rats.

In relation to the oxidative state, β-caryophyllene and copaiba oil at the highest dose reduced the protein carbonyl groups and ROS in the arthritic liver to levels close to the control values (Table III). In addition, at both doses, they increased GSH in the arthritic liver to levels close to the control values [Castro-Ghizoni et al., 2017]. These results strongly suggest that β-caryophyllene is responsible for the antioxidant action of copaiba oil in the liver of arthritic rats. In this regard, β-caryophyllene had higher inhibitory capacity on lipid peroxidation of liver microsomes than α-tocopherol and still much higher than α-humulene [Calleja et al., 2013]. On the other hand, at the lowest dose, only β-caryophyllene reduced the levels of ROS and GSSG in arthritic livers. However, copaiba oil was associated with harmful actions in the liver, a condition that should impair an effective antioxidant action [Castro-Ghizoni et al., 2017]. This fact is corroborated by the ineffective action of copaiba oil on oxidative stress in the plasma of arthritic rats, which could be improved by β-caryophyllene (430 $mg \cdot Kg^{-1}$).

β-Caryophyllene may decrease the oxidative stress by three different mechanisms: by decreasing the inflammatory process (1), by stimulating the endogenous antioxidant system (2), or by acting as direct free radicals scavenger (3). With respect to the mechanism (1), the activity of MPO is an indicator of polymorphonuclear cells infiltration and β-caryophyllene reduced it in the liver and plasma of arthritic rats. In addition to parameters verified in the present study, β-caryophyllene has been reported to downregulate the expression of cyclooxygenase 2 (COX 2), TNF-α, IL-6 and IL-1β in various *in*

vitro and animal models of neuroinflammation [Ojha et al., 2016; Guo et al., 2014; Cheng et al., 2014]. Recent studies reported that anti-inflammatory effects of β-caryophyllene involve activation of cannabinoid receptor 2 (CB2) and of the peroxisome proliferator-activated receptor-γ (PPARγ) pathway [Bento et al., 2011; Cheng et al., 2014]. In this regard, agonists of CB2 receptors have been reported to inhibit production of IL-6 and matrix metalloproteinase-3 in TNFα-stimulated fibroblast-like synoviocytes derived from the rheumatoid joints [Fukuda et al., 2014].

In addition, β-caryophyllene increased the hepatic content of GSH, one component of the endogenous antioxidant system (mechanism 2). Contributing to this finding, β-caryophyllene has been reported to induces nuclear translocation of nuclear factor erythroid 2-related 2 (Nfr2), which upregulates antioxidant defense genes, and improve the cellular GSH antioxidant system in C6 glioma cells and in rats with cerebral ischemia-reperfusion injury [Assis et al., 2014; Lou et al., 2016].

With respect to the mechanism (3), β-caryophyllene has been reported to present antioxidant action via free radical scavenging against hydroxyl radicals, superoxide anions and lipid peroxides [Ojha et al., 2016; Guo et al., 2014; Calleja et al., 2013]. The scavenging activity of β-caryophyllene has been explained by the structure presenting double rings which allow radical insertion on the ring of both olefinic systems favoring the generation of tertiary radicals and the allylic system of high stability (Fig. 1A) [Ojha et al., 2016]. Another point that deserves additional comment is the plasmatic antioxidant status. In arthritic rats, β-caryophyllene (430 $mg \cdot Kg^{-1}$) increased the antioxidant capacity of the plasma, where antioxidant enzymes and glutathione contribute poorly and it depends mainly on albumin thiol groups [Bracht et al., 2016]. In the present study, the plasmatic levels of albumin and thiols were reduced in the same proportion by arthritis and the treatment with β-caryophyllene did not increase them. Thus, it is possible that β-caryophyllene had improved the plasmatic antioxidant activity itself as a free radical scavenger. Therefore, it is probable that β-caryophyllene had improved the oxidative status of arthritic rats by means of all three mechanisms listed above.

The mitochondrial respiration and ROS production of treated rats were not modified by arthritis or β-caryophyllene and this point deserves a few comments. Mitochondria are the main site of ROS production in the cell and it

would be expected a higher ROS production in mitochondria isolated from arthritic livers, where the ROS content was higher. Similarly, it would be expected a smaller ROS production after β-caryophyllene treatment. In addition, β-caryophyllene exogenously added inhibited the mitochondrial ROS production in murine BV2 cells [Guo et al., 2014]. The same can be said about the respiration of isolated mitochondria, which is modified by changes in the redox state. The fact that no change occurred in the ROS production and respiration of isolated mitochondria (Fig. 3) does not disprove that the phenomenon occurs *in vivo* because incubations of isolated organelles do not reproduce these conditions, as inflammatory mediators and β-caryophyllene, for example, are absent. The same phenomenon was already reported for arthritic rats and for copaiba oil treatment [Castro-Ghizoni et al., 2017; Comar et al., 2013].

The reduced gluconeogenesis from L-lactate in the perfused livers of arthritic rats was already previously verified, including from other substrates, and it has been reported to occur due to lower activity of gluconeogenic key enzymes [Fedatto et al., 1999]. The treatment of arthritic rats with β-caryophyllene was practically without effect on the reduced gluconeogenesis in arthritic rats. The same effect was verified on the gluconeogenesis of arthritic rats treated with copaiba oil. The oil in additionally reduced the gluconeogenesis in the liver of control rats [Castro-Ghizoni et al., 2017]. The latter was attributed to harmful effects of the oil, which was associated with an impairment of the liver function due to hepatic cholestasis [Castro-Ghizoni et al., 2013]. However, β-caryophyllene did not modify the gluconeogenesis of control rats in the present study, what allows conclude that the sesquiterpene do not impair the liver function. In this regard, plasmatic markers of liver damage were not changed or even increased by the treatment of control rats with β-caryophyllene, what reinforces the hypothesis that, unlike copaiba oil, it is not associated with hepatotoxicity. Hepatoprotective action has been attributed to the sesquiterpene [Calleja et al., 2013; Varga et al., 2018]. A justification for the toxic action of oil is that, cytotoxic activity has been attributed to the kaurenoic and hardwickiic acids, which are among the main diterpenes in copaiba oil (*C. reticulata*) and, therefore, they could be associated with the harmful actions of the oil [Soares et al., 2013; Veiga Jr et al., 2007].

β-Caryophyllene was not associated with hepatotoxicity, but it was

practically without effect on the alterations in the number and size of hepatocytes in arthritic rats. The greater area of the hepatocytes observed in consequence of arthritis arises probably from the swelling of these cells. By occupying more space, fewer hepatocytes are found per unit liver area, a condition that increases the size and weight of the liver (swollen liver), as indeed found in the present study (Table I).

Cellular swelling is an intracytoplasmic accumulation of water due to the incapacity of the cells to maintain the fluid homeostasis and it is reported to be an alteration resulting as a response to nonlethal injuries and stressors [Del Monte, 2005]. It is easy to be observed in parenchymal organs, particularly liver, where it can occur including as normal adaptive response involved in the modulation of the hepatic metabolism [Del Monte, 2005]. Accumulation of biomolecules in the cytosol is normally associated with hepatocytes enlargement, a phenomenon known as “cloudy swelling” [Del Monte, 2001]. In wasting chronic inflammation, the liver shifts protein synthesis away from albumin and toward an increased production of acute-phase proteins. The latter do not accumulate within the hepatocytes, nevertheless, their synthesis contributes to the marked increase in the uptake of muscle-derived amino acids observed in swollen hepatocytes [Del Monte, 2001]. This condition is verified even in rheumatoid arthritis, which in long-term can cause cachexia [Walsmith & Roubenoff, 2002]. The liver enlargement is not an appreciated feature of rheumatoid arthritis, however, hepatomegaly is not necessarily related to severe complications of the disease [Tiger et al., 1976].

β-Caryophyllene did not reduce the hepatocytes and liver enlargement in arthritic rats, but it also did not increase the body weight and plasmatic albumin levels, which show that the chronic inflammation inherent to this model of arthritis was not improved completely. Nevertheless, the adjuvant-induced arthritis is severe and in the literature, it is reported that even ibuprofen, indomethacin and dexamethasone treatment improved the liver and body weight [Bendele et al., 1999]. In addition, the reduced gluconeogenesis in arthritic rats seems to occur as consequence of hepatocyte swelling, which in long-term has been reported to decrease the transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 1,6 bisphosphatase [Meijer, 2000].

In summary, the results of the present study revealed that β-

caryophyllene presents articular and systemic anti-inflammatory activities on adjuvant-induced arthritis in rats and that it was effective in decreasing oxidative stress in the liver. These actions were at the same extension of copaiba oil (*C. reticulata*) on arthritic rats, in previous work and, therefore, β-caryophyllene seems to be responsible for these copaiba oil actions. β-Caryophyllene shows in addition plasmatic antioxidant action and hepatoprotective activity. On the other hand, β-caryophyllene (and copaiba oil) did not improve the adjuvant-induced swelling of hepatocytes, decreased gluconeogenesis, hepatomegaly and low body weight of arthritics rats, however, even nonsteroidal anti-inflammatory drugs did not improve some these modifications. Nevertheless, unlike on the study with copaiba oil, these parameters were not modified in healthy treated rats and, therefore, β-caryophyllene was not associated with hepatotoxicity. With this in view, β-caryophyllene may be a good candidate as an adjuvant to potentiate the effects of conventional treatment to rheumatoid arthritis or even a starting point for new anti-inflammatory drugs development.

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List of abbreviations

Abbreviations: ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione; TNF-α, tumoral necrosis factor alpha; IL-1 and 6, interleukin 1 and 6; NFkB, nuclear factor kappa B; SOD, superoxide dismutase; MPO, myeloperoxidase; COX 2, cyclooxygenase 2; CB2 receptor, cannabinoid receptor 2; Nfr2, nuclear factor erythroid 2-related 2; TAC, total antioxidant capacity; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

Competing interests

The authors declare that no competing interest exists and that all approved the final manuscript.

Authors' contributions

JFC and AB conceived and designed the experiments. CABA and APAS induced arthritis and treated the animals. APAS and FMSS performed the experiments with inflammation. APAS and CVCG performed the liver perfusion experiments. MRMN and CVCG performed the liver histological experiments. APAS, ABSN, and LB performed the oxidative status and mitochondrial experiments. JFC wrote the paper. AB reviewed the paper. All authors have read and approved the final version of the paper.

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TABLE I. **Inflammatory parameters and organ and body weights.** Initial paw volume was 1.43 ± 0.03 ml; Δvol of paws=volume at day 18 - initial volume (mL). Initial body weight (at day 0) was 202 ± 4 g; Liver and lymph nodes (LD; right + left) weights are expressed as g and mg·(100 g animal weight)$^{-1}$. C, controls treated with saline; Ccorn, controls treated with corn oil; Cβ 215 and 430, controls treated with 215 and 430 mg·Kg^{-1} β-caryophyllene; A, arthritis (saline); Acorn, arthritis (corn oil); Aβ 215 and 430, arthritis (215 and 430 mg·Kg^{-1} β-caryophyllene); AIBU, arthritic rats treated with ibuprofen (30 mg·Kg^{-1}).

Parameter	Groups								
	C	Ccorn	Cβ 215	Cβ 430	A	Acorn	Aβ 215	Aβ 430	AIBU
Δvol (injected paw)	---	---	---	---	4.4±0.3^{a}	4.4±0.3^{a}	3.1±0.4^{b}	3.0±0.3^{b}	2.5±0.1^{b}
Δvol(non-injected)	---	---	---	---	2.3±0.1^{a}	2.4±0.1^{a}	1.8±0.2^{a}	1.0±0.3^{b}	1.2±0.2^{b}
Arthritic score	---	---	---	---	5.0±0.0^{a}	5.0±0.0^{a}	4.2±0.4^{ab}	4.4±0.4^{ab}	3.8±0.3^{b}
Body weight (g)	284±12^{a}	274±10^{a}	301±16^{a}	286±7^{a}	202±4^{b}	190±6^{b}	203±9^{b}	197±6^{b}	208±12^{b}
Liver	2.7±0.0^{a}	2.8±0.1^{a}	2.7±0.1^{a}	3.0±0.1^{a}	4.3±0.1^{b}	4.1±0.2^{b}	4.3±0.3^{b}	4.4±0.2^{b}	4.4±0.2^{b}
Popliteus LD	9.4±1.6^{a}	8.8±0.9^{a}	12.1±0.8^{a}	7.3±0.6^{a}	116±27^{b}	95±14^{bc}	65±11^{cd}	57±14^{d}	60±16^{cd}
Inguinal LD	16.1±1.0^{a}	15.6±1.0^{a}	15.8±1.1^{a}	12.9±0.6^{a}	90±16^{b}	92±10^{b}	96±17^{b}	53±9^{c}	65±8^{bc}
	Initial (at day 0)				blood leukocytes (at day 18)				
Total leukocytes (x10^{3}) (mm^{3})$^{-1}$	14.6±1.1^{a}				64.0 ±9.7^{b}	52.8±2.3^{bc}	38.7±5.2^{c}	40.4±2.8^{c}	38.7±4.6^{c}
PMN cells (%)	11 ± 2^{a}				70 ± 2^{b}	64 ± 5^{bc}	57 ± 6^{bc}	46 ± 10^{c}	62 ± 3^{bc}
					Articular leukocytes (hind left joint)				
Total leukocytes (x10^{4}) (mm^{3})$^{-1}$	---				19.0±4.6^{a}	14.5±0.7^{a}	4.0±1.4^{b}	4.8±1.3^{b}	4.7±1.3^{b}
PMN cells (%)	---				73 ± 10^{a}	72 ± 5^{a}	40 ± 9^{ab}	38 ± 8^{b}	64 ± 2^{ab}
					Articular leukocytes (hind right joint)				
Total leukocytes (x10^{4}) (mm^{3})$^{-1}$	---				10.2±3.2^{a}	11.1±3.1^{a}	8.3±4.8^{a}	7.4±2.3^{a}	2.5±1.0^{b}
PMN cells (%)	---				52±10^{a}	73±9^{a}	52±6^{a}	43±9^{a}	58±7^{a}

The data are the mean ± standard error of four to six animals. Values with different superscript letters in the same line are different (p <0.05).

TABLE II. **Hepatic antioxidant parameters and plasmatic markers of liver damage.** C, controls treated with saline; Cβ 215 and 430, controls treated with 215 and 430 mg·Kg^{-1} β-caryophyllene; A, arthritis; Cβ 215 and 430, arthritic rats treated with 215 and 430 mg·Kg^{-1} β-caryophyllene; AIBU, arthritic rats treated with ibuprofen (30 mg·Kg^{-1}). Liver parameters are referred per mg of protein.

	Groups						
Parameter	C	Cβ 215	Cβ 430	A	Aβ 215	Aβ 430	AIBU
	Liver parameters						
GSH (nmol·mg^{-1})	11.4 ± 0.4^{a}	10.7 ± 0.9^{a}	9.6 ± 0.9^{a}	4.2 ± 0.6^{b}	7.1 ± 1.8^{a}	8.5 ± 0.9^{a}	9.9 ± 1.3^{a}
GSSG (nmol·mg^{-1})	1.6 ± 0.2^{a}	1.8 ± 0.3^{a}	1.5 ± 0.5^{a}	4.0 ± 0.4^{b}	2.9 ± 0.6^{a}	1.7 ± 0.2^{a}	2.7 ± 0.4^{a}
GSH/GSSG ratio	7.4 ± 0.7^{a}	6.9 ± 1.9^{ac}	7.0 ± 1.3^{ac}	1.3 ± 0.2^{b}	2.6 ± 0.4^{bc}	5.0 ± 0.3^{ac}	4.0 ± 0.7^{c}
Catalase (mmol·min^{-1}·mg^{-1})	1.1 ± 0.1^{a}	0.9 ± 0.1^{a}	1.0 ± 0.1^{a}	0.25 ± 0.02^{b}	0.24 ± 0.03^{b}	0.31 ± 0.04^{b}	0.25 ± 0.03^{b}
SOD (U·mg^{-1})	2.6 ± 0.1^{a}	2.5 ± 0.2^{a}	2.2 ± 0.2^{ab}	1.5 ± 0.2^{b}	2.0 ± 0.2^{ab}	2.2 ± 0.1^{ab}	2.2 ± 0.2^{a}
	Plasmatic parameters						
AST (U·L^{-1})	41.6 ± 3.3^{a}	45.5 ± 2.0^{a}	54.6 ± 4.6^{a}	81.8 ± 8.9^{b}	41.0 ± 2.8^{a}	47.3 ± 2.6^{a}	61.7 ± 6.7^{a}
ALT (U·L^{-1})	32.7 ± 1.5^{ab}	39.0 ± 2.9^{a}	37.5 ± 6.6^{a}	29.4 ± 1.5^{ab}	22.0 ± 2.6^{b}	38.1 ± 3.9^{a}	34.0 ± 4.1^{ab}
ALP (U·L^{-1})	51.4 ± 6.1^{a}	50.9 ± 4.2^{a}	37.9 ± 8.4^{a}	125.6 ± 21.7^{b}	58.9 ± 7.2^{a}	53.5 ± 7.1^{a}	48.3 ± 10.5^{a}
Total protein (g·dL^{-1})	5.2 ± 0.2^{a}	5.6 ± 0.3^{a}	5.2 ± 0.2^{a}	6.0 ± 0.2^{a}	6.1 ± 0.4^{a}	6.0 ± 0.2^{a}	5.8 ± 0.2^{a}
Albumin (g·dL^{-1})	2.1 ± 0.1^{a}	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}	1.5 ± 0.1^{b}	1.4 ± 0.1^{b}	1.4 ± 0.2^{b}	2.1 ± 0.1^{a}

The data are the mean ± standard error of three to eight animals. Values with different superscript letters in the same line are different (p < 0.05).

TABLE III. **Comparative effects of β-caryophyllene and copaiba oil (*C. reticulata*) on the inflammatory and oxidative status of arthritic rats**. Values are % of the increase (+) or decrease (-) of the parameters caused by the supposed treatment in relation to the arthritic group (Arthr). When the increase or decrease of a parameter is represented as 100%, it means that the treatment was effective in restoring normal values (statistically equal to healthy animals). Animals were orally treated with 215 and 430 mg·Kg^{-1} β-caryophyllene (Aβ 215 and 430) or 30 mg·Kg^{-1} ibuprofen (AIBU) as described in Methods. The doses of the sesquiterpene are equivalent to the volume of β-caryophyllene in the doses of copaiba oil used in previous study, 0.58 and 1.15 g·Kg^{-1} copaiba oil (Cop 0.58 and 1.15). Values for β-caryophyllene were calculated from the results of the present work and values for copaiba oil were calculated from the results of a previous study [Castro-Ghizoni et al., 2017]. ↑ and ↑↑ mean, respectively, moderate and accentuated increases; --- means "not measured". TAC, total antioxidant capacity.

Parameter	Arthr	Aβ 215	Aβ 430	Cop 0.58	Cop 1.15	AIBU
Edema (injected paw)	↑↑	-30%	-30%	No	-30%	-45%
Edema (contralateral paw)	↑↑	No	- 50%	-45%	-50%	-50%
Popliteus lymph nodes (swollen)	↑↑	-45%	-50%	-50%	No	-50%
Total leukocytes (blood)	↑↑	-40%	-40%	---	---	-40%
MPO activity (plasma)	↑↑	-35%	-35%	-30%	---	-35%
MPO activity (liver)	↑	No	-100%	No	No	-100%
GSH content (liver)	↓↓	+100%	+100%	+100%	+170%	+100%
ROS content (liver)	↑↑	-100%	-100%	No	-100%	-30%
Carbonyls groups (liver)	↑↑	No	-100%	No	-100%	-100%
Carbonyl groups (plasma)	↑	-20%	-100%	No	No	-100%
TAC (plasma)	↓↓	No	+100%	No	No	No

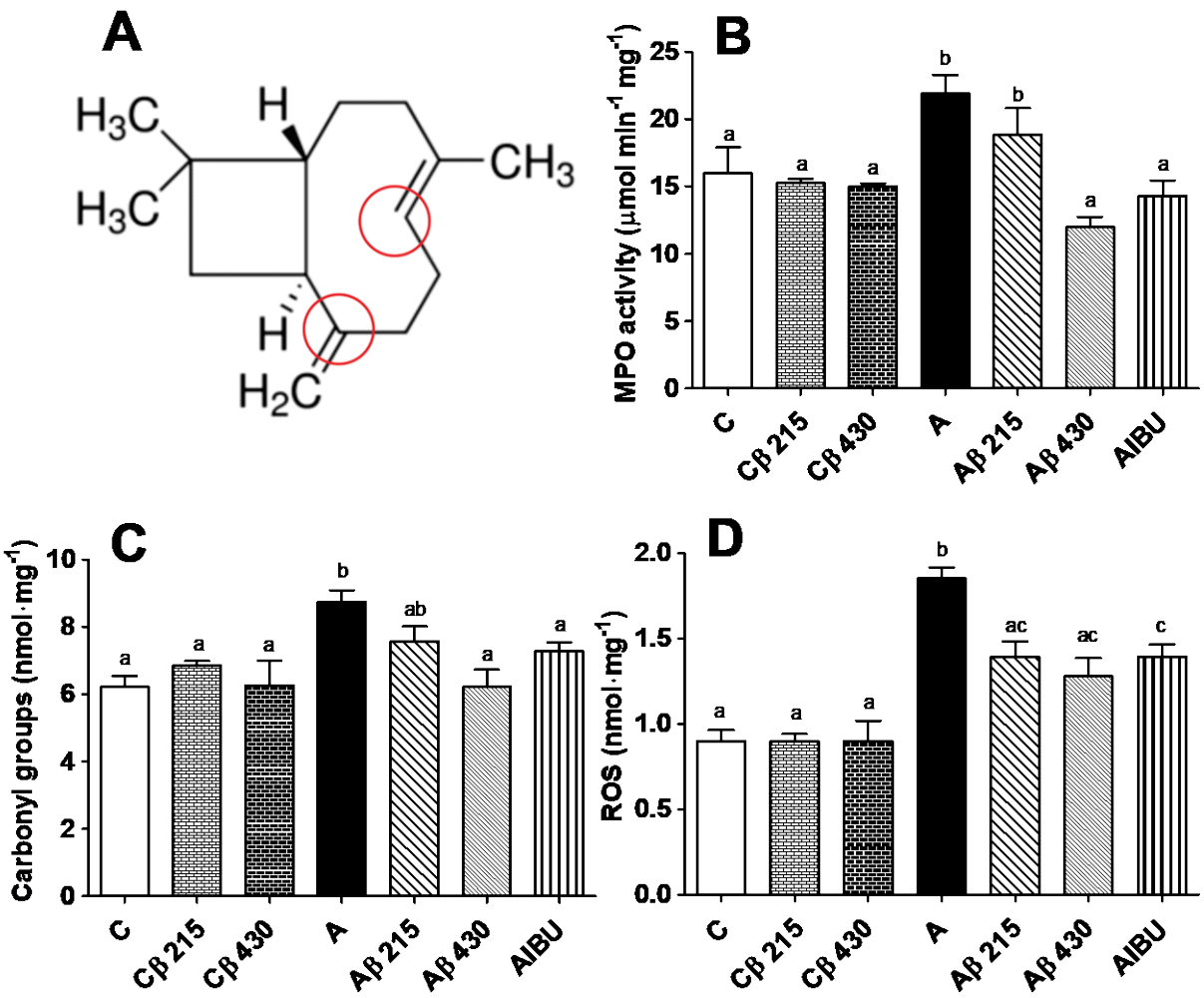


Fig. 1. **Chemical structure and effects of β-caryophyllene on the oxidative state in the liver of control and arthritic rats. (A)** Chemical structure of β-caryophyllene (red circles show sites for antioxidant action). The image was constructed using the program CorelDRAW® Graphics Suite X7 (Corel Corporation) and based on image from Ojha et al [2016]; **(B)** Liver myeloperoxidase activity; **(C)** Hepatic levels of protein carbonyl groups; **(D)** Hepatic levels of Oxygen reactive species (ROS). C, controls treated with saline; Cβ215 and 430, controls treated with 215 and 430mg·Kg^{-1} β-caryophyllene; A, arthritic rats treated with saline; Aβ215 and 430, arthritic rats treated with 215 and 430mg·Kg^{-1} β-caryophyllene; AIBU, arthritic rats treated with ibuprofen (30 mg·Kg^{-1}). Data represent the mean ± SEM of three to ten animals. Values with different superscript letters are statistically different (P <0.05).

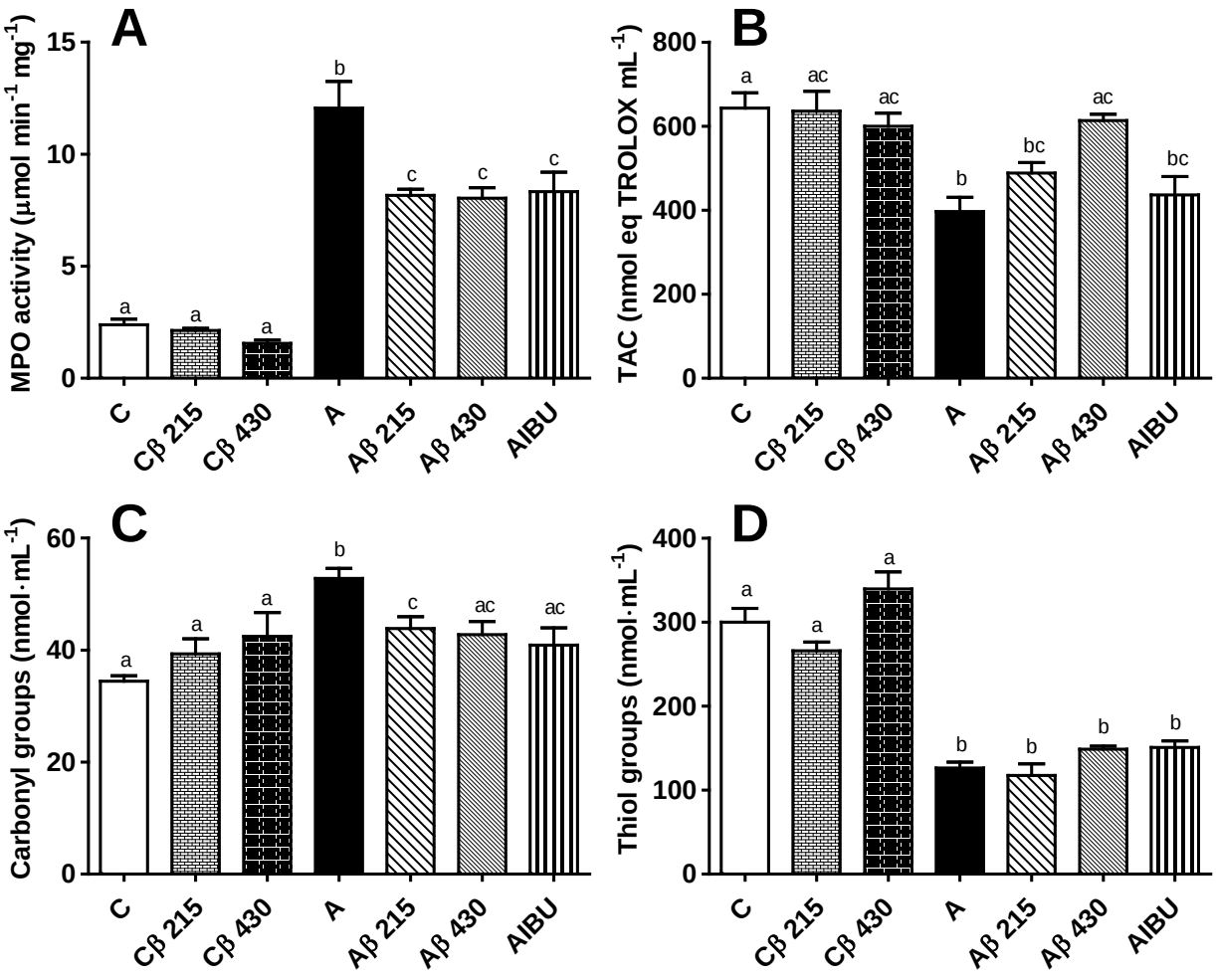


Fig. 2. **Effects of β-caryophyllene on the oxidative state in the plasma of control and arthritic rats. (A)** Plasmatic myeloperoxidase (MPO) activity; **(B)** Plasmatic total antioxidant capacity (TAC); **(C)** Plasmatic protein carbonyl groups; **(D)** Plasmatic sulfhydryl groups (thiols). C, controls treated with saline; Cβ215 and 430, control treated with 215 and 430mg·Kg^{-1} β-caryophyllene; A, arthritic rats treated with saline; Aβ215 and 430, arthritic rats treated with 215 and 430mg·Kg^{-1} β-caryophyllene; AIBU, arthritic rats treated with ibuprofen (30 mg·Kg^{-1}). Data represent the mean ± SEM of three to eight animals. Values with different superscript letters are statistically different (P <0.05).

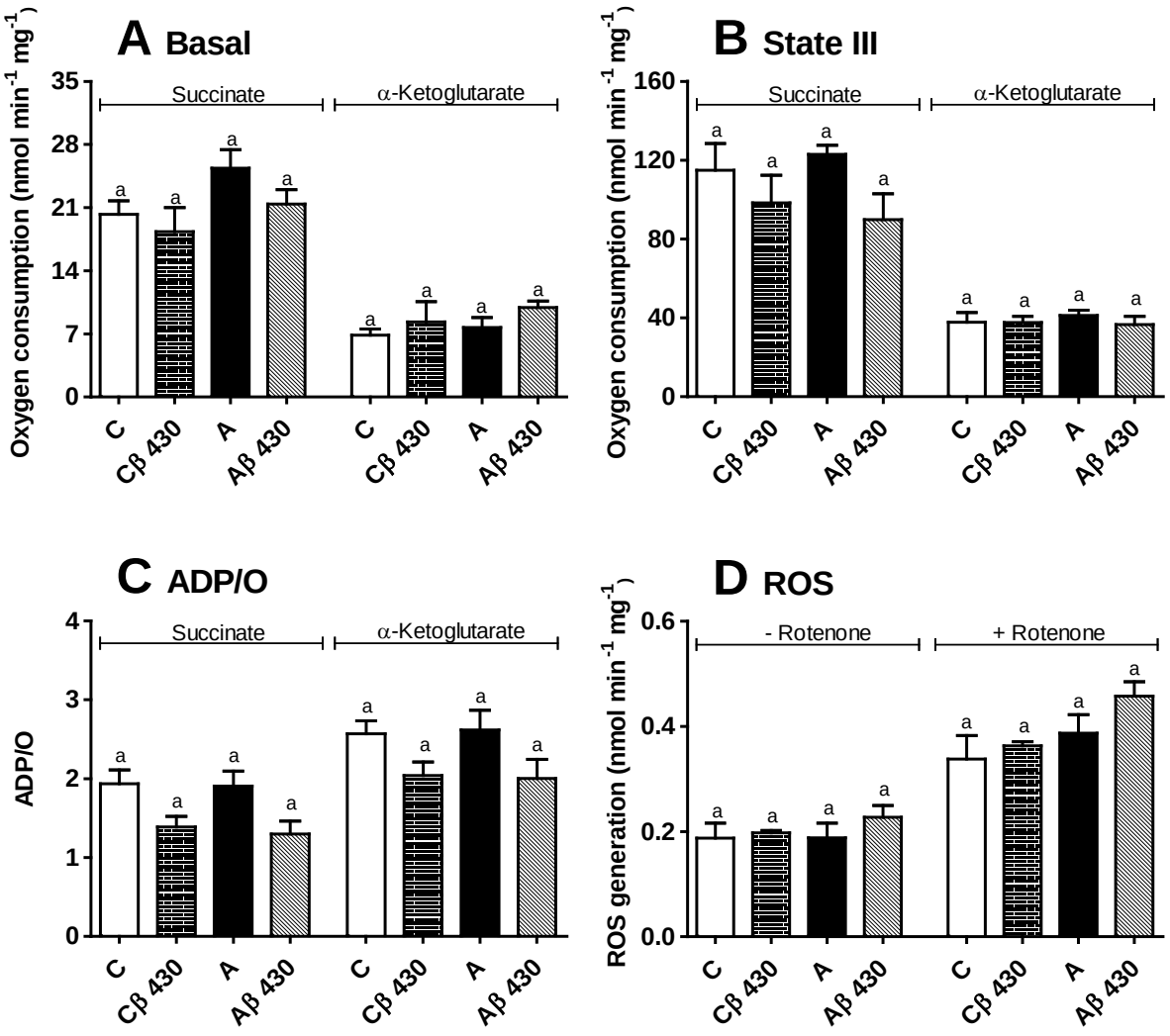
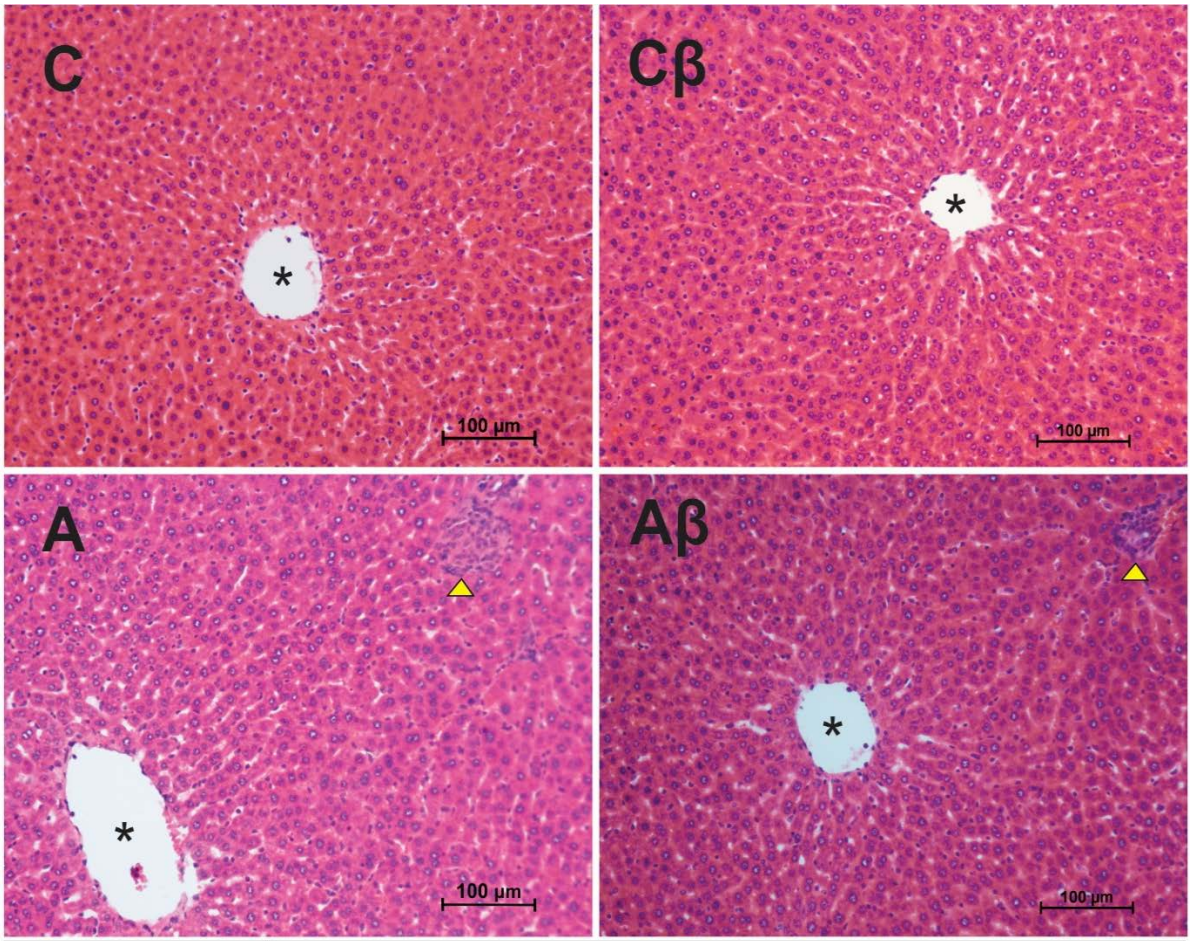


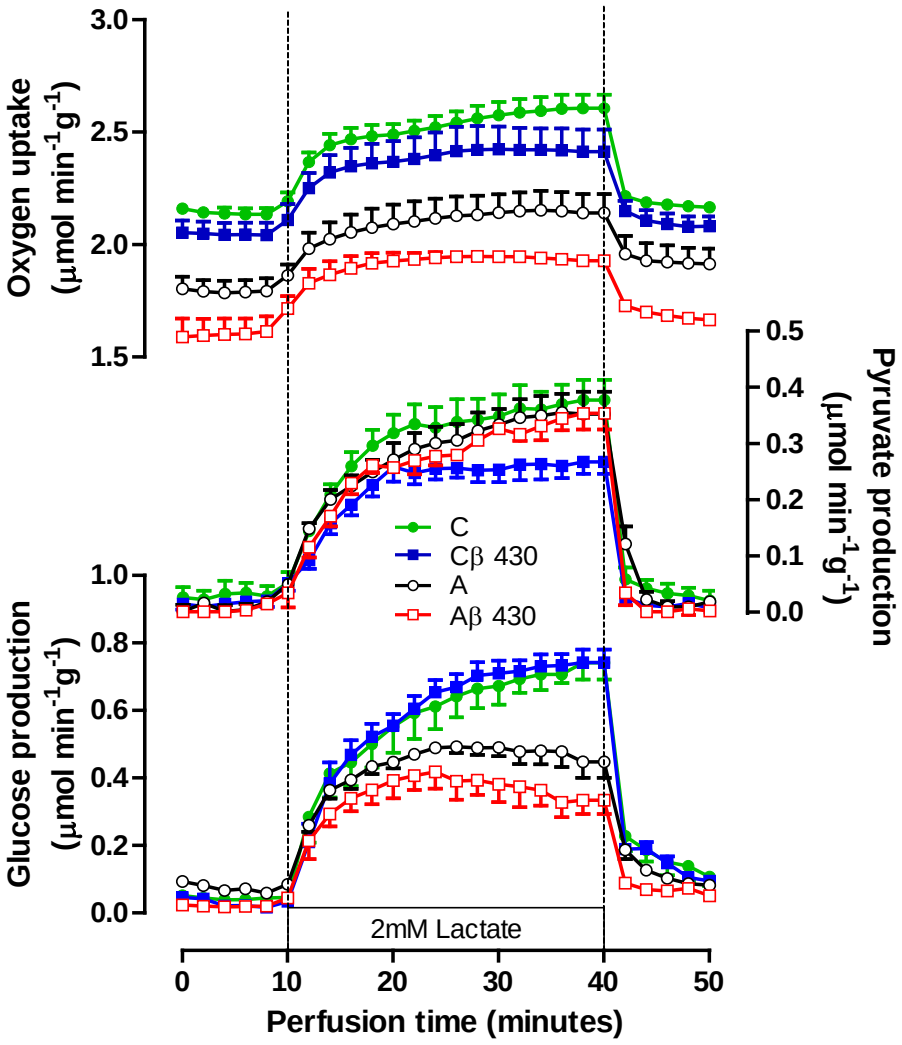
Fig. 3. **Effects of β-caryophyllene treatment on reactive oxygen species (ROS) production and respiratory activity of intact isolated hepatic mitochondria.** For respiratory activity, mitochondria (1.0 mg·mL^{-1}) were incubated at 37ºC in a closed oxygraph chamber containing 2 mL of the reaction medium. Mitochondrial respiration is driven by α-ketoglutarate or succinate in the absence **(A; basal)** and presence **(B; state III)** of 0.125 mM ADP was followed polarographically. **(C)**, ADP/O ratio. Mitochondrial ROS production **(D)** driven by succinate in the presence and absence of rotenone was followed by spectrofluorimetrically as described in Methods. C, controls (saline); Cβ 430, control treated with 430mg·Kg^{-1} β-caryophyllene; A, arthritic rats (saline); Aβ 430, arthritic rats treated with 430mg·Kg^{-1} of β-caryophyllene; Data represent the mean ± SEM of three to six animals. Values with equal superscript letters are not statistically different (p <0.05).



Parameter	Groups			
Hepatocyte	C	Cβ	A	Aβ
Number $(mm^2)^{-1}$	3410 ± 15^a	3380 ± 74^a	2619 ± 79^b	2751 ± 76^b
Area (μm^2)	151 ± 4^a	155 ± 5^{ab}	178 ± 10^b	171 ± 3^{ab}

Values with different superscript letters in the same line are different ($p<0.05$).

Fig. 4. **Photomicrographs of liver sections and morphometric analysis of control and arthritic rats treated or not with β-caryophyllene.** C, controls (saline); Cβ, controls treated with β-caryophyllene at the dose of 430 $mg \cdot kg^{-1}$; A, arthritic rats (saline); Aβ, arthritic rats treated with β-caryophyllene at the dose of 430 $mg \cdot kg^{-1}$. Hematoxylin-eosin staining (x200). Scale = 100µm. Number of hepatocytes per mm^2. The data represent the mean ± standard error of five animals. *Indicates the center lobular vein. The arrow indicates an inflammatory focus.



Groups	Parameter (µmol·min^{-1}·g^{-1})		
	Glucose production	Pyruvate production	Oxygen uptake
C	0,66 ± 0,04^{a}	0,33 ± 0,03^{a}	0,45 ± 0,06^{a}
Cβ 430	0,71 ± 0,04^{a}	0,27 ± 0,02^{a}	0,40 ± 0,05^{a}
A	0,39 ± 0,02^{b}	0,34 ± 0,04^{a}	0,36 ± 0,04^{a}
Aβ 430	0,34 ± 0,05^{b}	0,33 ± 0,01^{a}	0,34 ± 0,05^{a}

Fig. 5. **Effects of β-caryophyllene on the liver gluconeogenesis of control and arthritic rats.** Time course of gluconeogenesis (upside). Livers from fasted rats were perfused with Krebs/Henseleit-buffer. L-lactate was the substrate. The values in steady-state (bottom) were calculated as [final values at 40 min] - [basal rates at 10 min]. C, control (saline); Cβ 430; control treated with 430 mg·Kg^{-1} β-caryophyllene; A, arthritis (saline); Aβ 430, arthritic rats treated with 430 mg·Kg^{-1} β-caryophyllene. Data point represents the means ± SEM of three to five livers. Values with different superscript letters are different (p <0.05).